Duckweed (*Lemna minor* D0158): a promising protein source for food security

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Duckweeds, recognized as the fastest growing aquatic flowering plants, exhibit substantial biomass production. Recently, they have been emerged as a potential industrial crop for efficient and eco-friendly protein production and nutrient recovery compared to conventional crops. The objective of the study was to determine the biomass accumulation rate, protein content and amino acid analysis of duckweeds (*Lemna minor* D0158). We cultured *L. minor* D0158 from its preserved state in the gene bank into both aseptic (*in-vitro*) and open condition at controlled room. The medium was Hoagland Solution (HS) mixed with sucrose in *in-vitro* condition while 1/5 concentration of HS (without sucrose) was in the open condition. Subsequently, we determined the dry biomass growth by calculating the weight difference adopting the Bergmann Method, and then calculated the protein percentage following the Kjeldahl Method. Moreover, the amino acid profiling was obtained using the Hydrolysis and Liquid Chromatography Technique. Later on, we compared the results with those of the other duckweeds and soybean together with the FAO requirements. We found that *L. minor* D0158 possessed the dry biomass growth rate of 6.72 g/m^2/d with 33.13% protein content within 7-day cultivation period. Moreover, it exhibited significantly higher levels of branched chain amino acids (BCCAs) than soybean and met the FAO requirements. However, methionine (Met) content was found to be slightly low. The study suggested that *L. minor* D0158 might offer a sustainable solution for protein food security in future.

Keywords: Aquatic plant, biomass, Essential Amino Acids (EAAs), protein, sustainable protein source.

Globally, approximately 8.9% of the population, equivalent to 690 million people, suffer from hunger with an annual increase of 10 million and a cumulative rise of nearly 60 million over five years (FAO, 2020). Besides, food security is significantly more impacted by the prevalence of socioeconomic problems like extreme poverty, unemployment, and acute malnutrition (Fatema & Kaur, 2023). Addressing the challenge of meeting the growing demand for wholesome and nourishing food without threatening the natural environment is paramount (Langyan *et al*., 2022; Rini *et al*., 2022). To ensure ample supply of affordable, nutritious food, new and innovative agricultural and food systems must be explored (Vu *et al*., 2020; Xu *et al*., 2021). In this context, the exploration of plant-based proteins holds significance for

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their ease of availability, affordability, and environmentally friendly attributes. Plant-based protein can contribute to reduce the cost of livestock feed by partially replacing animal origin protein and major food crops as the demand for resource consuming animal-based protein further limits food obtainability (Shepaon *et al*., 2018). Notably, soybean meal stands out as a valuable protein source among ingredients frequently included in animal feed mixtures (Parrini *et al*., 2023). Despite being extensively cultivated in Brazil, USA, Argentina, China, and India for livestock feeds and biofuel, its dominance in the food and feed market poses a threat to the forest management and staple food production (Smaling *et al*., 2008; Hans, 2019). To address these challenges, the utilization of the fastest growing aquatic plants such as "duckweeds" (see Figure 1) can be valuable alternatives as they offer higher productivity and are cost effective compared to soybean (Appenroth *et al*., 2017). In fact, soy-protein dominates both the food and feed market, and the cultivation of soybean and its consumption presents massive threats to climate, water resources and the habitats of wildlife (Smaling *et al*., 2008).

Figure 1: Duckweeds (*Lemna minor*) growing in natural habitat (around the swamp of Rara Lake, Mugu district, Nepal).

Duckweeds (family Lemnaceae) are known as "aquatic model plants" in both academic and industrial research before 1990s, and are emerging with increased attention after 2000s (Zhao *et al*., 2012; Oláh *et al*., 2023). The first International Conference on Duckweed Research and Application (ICDRA) was held in Chengdu, China in 2011 followed by a number of conferences globally. Recently, it is going to be held in Thailand in 2024. Currently, the duckweeds offer a novel perspective on physiology and metabolic strategies for both academic research and applications (Acosta *et al*., 2021). Various duckweeds are extensively employed for pollutant recovery and phytoremediation (Chen *et al*., 2018; Ekperusi *et al*., 2019). Optimizing wastewater treatment yields advantages for society, ecology, and economy, and is crucial to fulfilling the 2030 Agenda for Sustainable Development (United Nations [UN], 2018).

Duckweeds are gluten-free and are rarely found to contain lectin which is a major component of soy beans. Lectin is a well-known anti-nutrient that binds to glycoprotein receptors on intestinal mucosal epithelial cells during nutrient absorption (Liener, 1994). Duckweeds retain less phytate than soybean meal (Rojas *et al*., 2014). Many plant proteins require thermal processing, such as cooking, extrusion, roasting, and streaming to reduce anti-nutrients and improve nutrient absorption (Hamad *et al*., 2019). Conversely, duckweeds can be used as food for humans without undergoing such an expensive and energy-intensive processing (Xu *et al*., 2021). Duckweeds have traditionally been consumed as human food particularly in Thailand and neighboring countries under the name “Khai-nam” which literally means “eggs of the water” or “possible source of inexpensive protein” (Bhanthumnavin & McGarry, 1971). All the five genera of duckweeds, namely *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia* do not have any detectable cytotoxic or anti-proliferative effect on the human cell lines like HUVEC, K-562, and HeLa, which is the first and crucial information to be accepted as human food (Sree, *et al*., 2019). Similarly, the advantage

of duckweed over other protein-rich plants of a similar nature is its ability to thrive in a wide range of environmental factors, such as nutrients, light, pH, and temperature (Fiordelmondo *et al*., 2022).

Among the duckweed genera, *Lemna* comprises 12 species with wider distribution and occurrence (Bog *et al*., 2019). *Lemna* contains one stabilizing root and three or four fronds, which are leaf-like structures. The other advantage of the genus *Lemna* is it can be grown in freshwaters at all seasons over a wide temperature range (Landolt, 1986; Zhao *et al*., 2014). Additionally, *Lemna* has proven to be a reliable source of protein supplements to both the human as well as animal feed (Kaplan *et al*., 2018).

This study aimed to estimate biomass accumulation rate, protein content, and amino acid profiling of *L. minor* D0158, one of the selected strains available in the Duckweed Gene Bank of Chengdu Institute of Biology, Chinese Academy of Sciences (CIB-CAS), China. We have compared the characteristics of *L. minor* D0158 with that of soybean, a prominent terrestrial plant protein source. This study provides insights to establish duckweed (*L. minor* D0158) as a sustainable and novel source of plant protein, and functional food which can contribute food and nutritional security in future.

Materials and methods

The study was conducted in the Duckweed Gene Bank of Chengdu Institute of Biology, Chinese Academy of Sciences (CIB-CAS), Chengdu, China, during July, 2019–September, 2019.

Sources of Duckweed

L. minor D0158, identified as one of the suitable germplasm in terms of both protein content and biomass growth rate in the CIB-CAS preserved in Murashige and Skoog (MS) solid medium, composed of 0.7% agar and 0.1% sucrose.

Growth medium

The composition of Stock Solution to prepare the Hoagland Solution (HS) included: i) Macro-nutrients ($Ca(NO_3)_2.4H_2O$: 59 g/L, KNO_3: 75.76 g/L, KH_2PO_4: 34 g/L, HCl 6M: 6 mL); ii) $MgSO_4.7H_2O$: 200 g/L; iii) EDTA /$C_{10}H_{16}N_2O_8$: 9 g/L, KOH 6M: 8 mL; iv) Tartaric Acid/$C_4H_6O_6$: 3 g/L; v) $FeCl_3.6H_2O$: 5.4 g/L; and vi) Micro-nutrients (H_3BO_3: 2.86 g/L, $ZnSO_4.7H_2O$: 0.22 g/L, $Na_2MoO_4.2H_2O$: 0.12 g/L, $CuSO_4.5H_2O$: 0.08 g/L, $MnCl_2.4H_2O$: 3.62 g/L, Table 1).

Table 1: Basic chemical composition of Hoagland Solution (HS)

S. N.	Stock solution	10 L (1/5 strength)	mL/L (full strength)	Remarks
1.	Macro-nutrients	40 mL	20	5.2 pH maintained using 20% HCl or 40% NaOH
2.	$MgSO_4.7H_2O$	5 mL	2.5	
3.	EDTA	2 mL	1	
4.	$FeCl_3.6H_2O$	2 mL	1	
5.	Micro-nutrients	2 mL	1	
6.	Tartaric Acid	2 mL	1	
7.	Sucrose	---	1.5	

Primary growth activation in aseptic condition

We conducted two phases of experiment. In order to obtain the optimum amount of biomass in the Growth Chamber (GZ-300-GSII, Shaoguan Guangzhi Technology Equipment Development Co., Ltd, Wuhan, China). In the first phase, 2–4 *Lemna* fronds were aseptically transferred into 100 mL Erlenmeyer Conical Flask containing 70 mL full strength HS and allowed to grow for two weeks. In the second experimental phase (after two weeks), 10–15 fully grown fronds were aseptically transferred into 150 mL Conical Flasks containing the same strength of HS. During this experimental phase, *Lemna* were allowed to grow for 3 weeks so as to obtain sufficient biomass for further experiment. In both the phases, we maintained the pH of the full-strength HS medium at 5.2 pH. Moreover, the condition was (25±2 °C temperature and photoperiod of 16:8 h i.e. 16 hours of light and 8 hours of darkness in

the growth chamber.

Biomass preparation and acclimatization in controlled room

Subsequently, the plant sample was first washed with running tap water gently for 3 minutes followed by deionized water 3 times to remove the nutrients of the medium, and then were dried in normal air dryer to remove water for 3 minutes. The duckweed (2.5 g) was then transferred into blue tray (17×11×5=935 cm) containing 700 mL of 1/5 strength HS to grow in normal growth condition (at 25±2 °C room temperature for a photoperiod of 16:8 h with a light intensity of 125 µmol/m^2/s) for 1 week. On the third day, 150 mL of 1/5 strength HS was added to replenish the loss of the medium through evaporation. This step was carried for acclimatization and sufficient biomass production for further experiment.

Determination of biomass accumulation rate and protein content

After one week, the plant sample was washed with the running tap-water gently for three minutes followed by deionized water three times to remove the nutrients of the medium. The sample was dried in normal air dryer for three minutes and weight was recorded. At this step, we placed 5 g initial biomass in a blue plastic tray (size: 17×11×5 cm) containing 700 mL of 1/5 strength HS (replacing nitrate macro-nutrient by adding 70 mg/L urea nitrogen, and 5.5 g/L anhydrous $CaCl_2$) and were allowed to grow in normal growing condition (16:8 h) photoperiod under 175 µmol/m^2/s light intensity at 25±2 °C temperature). The experiment was replicated three times (n=3). After one week, the grown *L. minor* D0158 was harvested by washing under running tap water for 3 minutes followed by rinsing with deionized water 3 times to remove the medium nutrients. After that, 12 g fresh weight was taken separately to calculate dry weight and protein percentage. Then, the harvested material was air-dried for 3 minutes and further dried for 24 hours at 60 °C temperature in hot air oven. The dried plant weight was recorded. The dry biomass accumulation rate (growth rate) was calculated using the Bergmann's Method (Bergmann *et al*., 2000). After that, the plant sample was powdered using a mortar and pestle. The Nitrogen concentration (%) was determined adopting the Kjeldahl Method (FOSS KJ 2200, Foss Corp, Sweden) to estimate protein (%) (GB/T, 1994). The equations used were as follows:

Biomass accumulation rate (g/m^2/d) = ΔW/s/t …… (1),

Where, ΔW = increase in dry weight (g);

s = calculated area (m^2) covered by the growth of *Lemna*; and

t = experimental time period in days (d)

Nitrogen (%) = HCl (mL) – Blank (mL) × 0.098 N (HCl concentration) × 14.007 × 100/dry weight of the sample (g) × 1000 …… (2); and

Protein (%) = Nitrogen (%) × 6.25 ……...…. (3)

Amino acid analysis

The quantitative determination of amino acid was carried out following the National Standard of the People's Republic of China (GB/T18246-2019) (GB/T, 2019). The sample (50 mg dry powder) was treated with hydrochloric acid (HCl 6: mol/L) and retained for 22 hours under vacuum conditions at 110±1 **℃** temperature. The resulting hydrolysis product was filtered and diluted with sodium citrate buffer for amino acid analysis with ninhydrin post-column derivatization ion exchange chromatograph (Sykam S-433D, chromatographic column LCA K06/Na). For tryptophan analysis, the duckweed sample (50 mg dry powder) was subjected to alkaline hydrolysis by lithium hydroxide solution (LiOH, 4 mol/L) under vacuum condition at 110±1 **℃** for 20 hours. Finally, the hydrolysis product was diluted with sodium citrate buffer and filtered for analysis by reversed-phase liquid chromatography (Agilent).

The amino acid profile in *L. minor* D0158 was examined and compared with the results of the previously studied duckweed species (Appenroth *et al*., 2017) and with other protein sources like legumes soybean (Ijeoma & Ubaka, 2019) and with the WHO-recommendations (WHO/

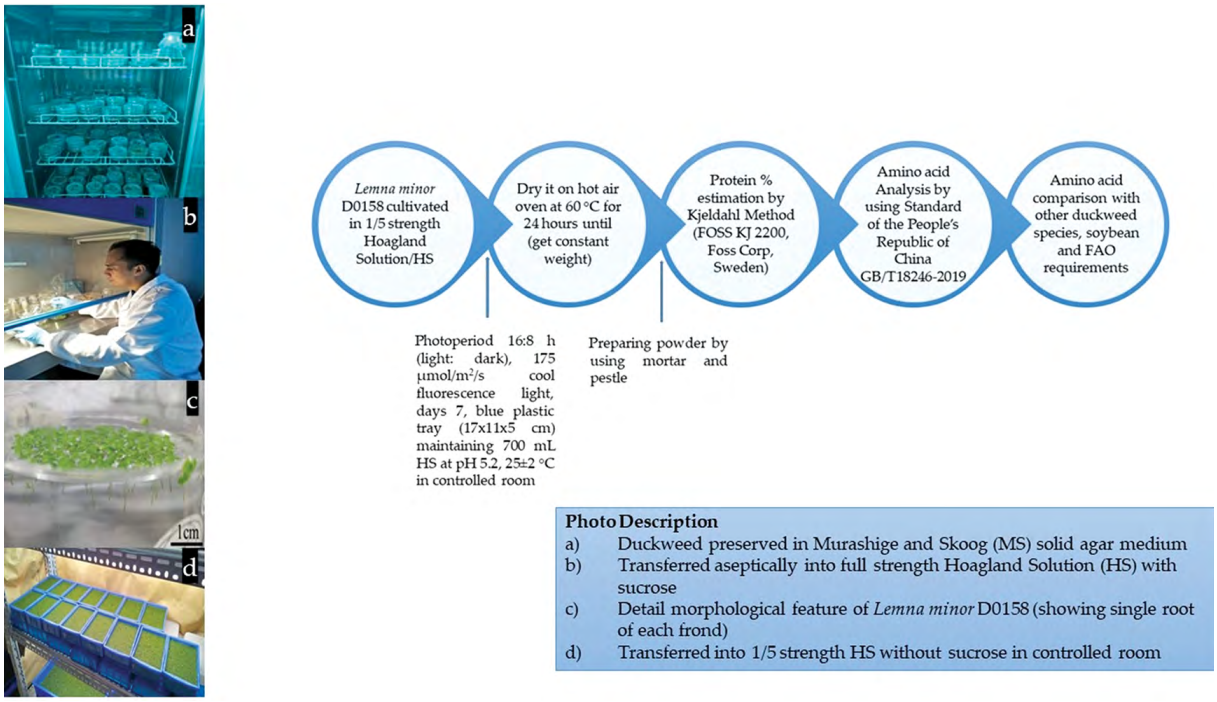

Figure 2: Flow chart of the study process.

FAO/UNU, 2007*)*. The overall study process is highlighted in Figure 2 below:

Statistical analysis

All the experiments were observed in triplicate (n=3), and the analyses were carried out in MS Excel Program 2013.

Results

L. minor D0158 in our investigation, displayed a dry biomass growth rate of 6.72 g/m²/d and protein content of 33.13% after 7 days cultured in 1/5 strength HS (see Table 2).

Table 2: Biomass growth rate and protein % at different time periods

Time	Biomass growth rate (g/m²/d)			
	n_1	n_2	n_3	Average
6 hr	-0.22	-0.22	-0.22	-0.22±0
1 d	0.82	0.84	0.85	0.84±0.01
3 d	2.49	2.52	2.55	2.52±0.03
5 d	4.00	4.04	4.08	4.04±0.04
7 d	6.62	6.72	6.82	6.72±0.10
9 d	6.35	6.41	6.48	6.41±0.06

	Protein %			
	n_1	n_2	n_3	Average
6 hr	31.16	32.26	33.35	32.25±1.09
1 d	28.08	28.70	29.32	28.70±0.62
3 d	28.7	29.00	29.30	29.00±0.30
5 d	30.91	31.28	31.65	31.28±0.37
7 d	32.30	33.13	33.95	33.13±0.82
9 d	31.14	31.95	32.75	31.94±0.80

In our study, amino acid profiling of *L. minor* D0158 revealed the quantities of aspartic acid (Asp), glycine (Gly), alanine (Ala), threonine (Thr), valine (Val), isoleucine (Ile), leucine (Leu), arginine (Arg), proline (Pro), phenylalanine+tyrosine (Phe+Tyr) were found to be higher than those in soybean (Ijeoma & Ubaka, 2019) and also higher than the FAO-recommended levels (see Table 3). In comparison to other previously studied duckweed species, *L. minor* D0158 strain exhibited similar or even higher essential amino acid (EAA) composition. Importantly, *L. minor* D0158 contains higher or comparable amounts of most amino acids present in soybean is presented in Table 3.

S. N.	Amino Acid % (g/100 g protein)	*L. minor* D0158	Soybean	FAO-recommendations
1.	Asp	18.97	11.53	-
2.	Thr	4.46	3.44	3.1
3.	Ser	4.76	5.54	-
4.	Glu	11.08	15.89	-
5.	Gly	5.46	3.37	-
6.	Ala	5.35	4.44	-
7.	Cys+Met	1.96	2.70	2.7
8.	Val	5.46	4.74	4.3
9.	Ile	4.29	3.60	3.2
10.	Leu	8.42	8.20	6.6
11.	Phe+Tyr	7.77	7.88	5.2
12.	Lys	5.90	6.36	5.7
13.	His	2.06	3.19	2
14.	Arg	8.14	6.71	-
15.	Pro	4.06	3.45	-

Table 3: Comparison of amino acid percentage of D0158 with those of soybean and FAO-recommendations (2011)

Discussion

Both protein percentage and dry biomass accumulation rate (growth rate) are important for any promising crop. The protein percentage and protein yields of common duckweed, soybean and other feed crops are presented in Table 4.

Table 4: The protein yields of duckweed and other feed crops (adapted from Hillman & Culley, 1978; Gijzen & Khondker, 1997)

Plant/crop	Yield (t dry wt/ ha/y)	Crude protein (% dry wt)	Relative protein production
Duckweed	17.60	37.00	100.00
Soybean	1.59	41.70	10.20
Alfalfa hay	4.37–15.69	15.90–17.00	11.40–38.30
Peanuts	1.60–3.12	23.60	5.70–11.30
Cotton seed	0.76	24.90	2.90

Note: The relative protein production of Duckweed is considered as: 100 units = 6.51 t dry wt/ha/year (Source: Iqbal, 1999).

The protein content in duckweeds (25–35% on dry biomass) depends upon the genotype and age of the species, growth conditions, such as light, temperature, nutrients, nutrient depth & agitation speed, and extraction methods (Rusoff *et al*., 1980; Casal *et al*., 2000). Regarding the biomass growth rate, our results were consistent with those reported by (Landolt, 1986; Leng *et al*., 1995). In another study performed by (Iqbal *et al*., 2019), it was found that the maximum growth rate of *L. minor* was 7.03 g/m^2/d in synthetic leachate when harvested in 10 days.

Duckweed prefers to consume urea nitrogen than nitrate nitrogen to synthesize protein content, which is due to low energy required by ammonium ion assimilation than nitrate assimilation (Tian *et al*., 2021). After seven days, our investigation yielded 33.13% protein, which is comparable to 33.56% found in a prior study (Landolt, 1986). Duckweed's protein content varied from 20% to 35% of its dry weight, surpassing that of cereals (6–15% protein) (Appenroth *et al*., 2017; Beukelaar *et al*.; 2018; Herawati *et al*., 2020). The primary protein (RuBisCO i.e. Ribulose 1, 5-Bisphosphate Carboxylase) in duckweeds is a good source of Essential Amino Acids (Goldberg, 2003). Due to its nutritional value, *in vitro* digestibility, and absence of allergies, RuBisCO protein is a good choice for a functional food (Chakrabarti *et al*., 2018; Yahaya *et al*., 2022).

Duckweed protein contains every amino acid that the human body needs. Together with non-EAAs, it includes all EAAs. Aspartic Acid (Asp) and glutamic acid (Glu) have regulatory roles in nutrition, energy metabolism and oxidative stress (Wang *et al*., 2017). In our study, the Asp was found to be 18.97%, significantly higher than of soybean (11.53%, Table 3). Asp serves as a precursor for amino acids such as methionine, threonine, isoleucine and lysine that regulate the secretion of crucial hormones (Chen *et al*., 2021). Additionally, Asp plays a role in controlling the production and synthesis of testosterone and luteinizing hormone (Topo *et al*., 2009). On the other hand, Glu serves as a flavor enhancer (Stryer, 1998). In our study, the Glu content in *L. minor* D0158 was found to be lower (11.08%) than in soybean (15.89%), which may decrease the palatability and taste preference of the

duckweed food product (Rangan & Barceloux, 2009). Furthermore, the aquatic clone of *L. minor* D0158 contain 5.9% Lysine/Lys, which is comparable to terrestrial soybean. Additionally, it plays a role in the production of carnitine, which is essential in fatty acid metabolism, and in the crosslinking of collagen polypeptides (Hall & da Costa, 2018). *Lemna gibba*, *Spirodela polyrhiza*, *Landoltia punctata*, and *Wolffia columbiana* were found to have the following average amino acid values (g/100 g of protein): 4.0 Lys, 3.6 Ile, 6.7 Leu, 0.9 Met, 7.3 Phe+Thr, and 4.4 Val. (Rusoff *et al*., 1980). In our study, we found 5.9 Lys, 4.29 Ile, 8.42 Leu, 7.77 Phe+Thr, and 5.46 Val. Moreover, the strain offers more advantages over most of the cereal crops (monocots) that contain inadequate levels of EAAs such as Lysine/Lys and Threonine/Thr (Vasal, 2020) whereas the content of Lys is low in corn and rice- 2.3 g/100 g and 3.2 g/100 g, respectively. However, duckweed contains only 0.9 methionine (Met) while corn and rice have high levels of the same- 3.1 and 3.4, respectively (Rusoff *et al*., 1980). Met (sulphur containing amino acid) is a precursor to homocysteine, cysteine, creatine, and carnitine as well as succinyl-CoA. Furthermore, Met has been shown in recent studies to control the innate immune system, metabolism, and digestive functions in mammals (Martínez *et al*., 2017).

Interestingly, the content of threonine in *L. minor* D0158 was 4.46%, higher than that of soybean (3.44%) and the FAO-recommendation of 3.1% for human consumption (FAO, 2011). Mostly, Thr is used as a substrate for the synthesis of proteins, especially mucin. Moreover, Thr has the ability to enter the catabolic pathway where it can be broken down into a number of vital byproducts that are essential to host metabolism, such as glycine, acetyl CoA, and pyruvate (Tang *et al*., 2021). Duckweed protein exhibits higher concentration of EAAs and is more similar to animal protein (Skillicorn *et al*., 1993). The EAA obtained in *L. minor* D0158 were similar to the FAO 2011 recommendation for human consumption except Met+Cys.

Our study showed *L. minor* D0158 clone had 37.18% EAA of the total amino acids, 17.15% glutamic acid of the total non-EAAs, and 47.48% Branched Chain Amino Acids (BCAAs) of the total EAAs. The BCAAs (Leucine/Leu, Isoleucine/Ile and Valine/Val) play key role in protein synthesis and muscle repair (Brestenský *et al*., 2015), particularly beneficial for athletes. The BCCA content in *L. minor* D0158 was found to be substantially higher (18.17% of the total amino acids), lower than in soybean (16.54%) and the FAO-recommendation 14.1% (FAO, 2011; Leser, 2013). In this regard, *L. minor* D0158 strain qualifies as an additional source of quality protein in terms of the total BCCAs percentage than soybean.

By 2050, there will be a two-fold increase in demand for animal-derived proteins due to the growing global population and rising meat consumption (FAO, 2011). Duckweed is a great option to meet the rising demand for animal-derived proteins worldwide because it can produce more protein for animal feed than traditional land-grown crops like soybeans; duckweed can produce 5-10 times as much protein per area. Moreover, switching people to more sustainable plant-based protein will be a better option (Roman *et al*., 2021). Moreover, duckweeds face no competition with other food and cash crops for arable land, and can be easily harvested (Bhanthumnavin & McGarry, 1971; Hillman, 1961; Appenroth *et al*., 2015). Interestingly, the researchers from Italy noted that 20% replacement of duckweed (*L. minor*) in the standard feed of rainbow trout had no adverse effects on the fish (Fiordelmondo *et al*., 2022). Additionally, duckweed contributes a valuable supplementation of balanced amino acids to grains (corn, maize) for animal and human consumption. Increased consumption of duckweeds, as a novel source of the plant-based protein, may contribute to improve health of the people and long-term sustainability of food supply (Casavale *et al*., 2016). Therefore, the utilization of duckweed, especially *L. minor* D0158, holds promise for both food and feed, offering a sustainable alternative plant-based protein that support to mitigate the issues of climate change such as deforestation and, environmental degradation associated with large-scale cultivation of soybeans. Ultimately, the study can contribute to achieve the foundation of the Sustainable Development Goal (SDG) 2 (freedom from hunger) that covers end hunger,

achieve food security and improved nutrition and promote sustainable agriculture and SDG 12 (responsible production and consumption).

Conclusion

Our study found that *L. minor* D0158 showed the biomass growth rate of 6.72 g/m^2/d while the protein content was 33.13%. All the EAA contents in the duckweed were found to be comparable to those of soybean, meeting the FAO requirements. The clone *L. minor* D0158 demonstrates as a significant protein source, bringing higher levels of amino acids such as aspartic acid, glycine, BCAAs and threonine compared to soybean, with lysine content nearly equivalent and lower in Methionine (Met.). This research holds promise for large-scale production and promotion of *L. minor* D0158 to tap its advantageous features such as aquatic production, fast growth rate that enhance low cost and easier production. Therefore, the clone can be a solution for protein food security in future. However, further studies should focus on seasonal variability, protein digestibility, bioavailability, safety considerations, and beneficial health-related claims.

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Author's Contribution Statement

Conceptualization, F. Y., T. L. and R. B.; methodology, R. B. and T. L., validation, T. L., D. A. P. and F.Y.; investigation, R. B.; data curation, D. A. P., T. L. and G. L.; writing-original draft preparation, R. B.; Writing-Review and Editing, D. A. P., J. Y. L., Y. Z. L., R. B.; visualization, D. A. P., Supervision, Z. H. and F.Y., Funding Acquisition, Z. H., F. Y., T. L., D. A. P., and H.T.F. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data used in this study are accessible upon request to the corresponding author.

Conflicts of interest

The authors declare no conflict of interest.

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